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Numbers and ratios of X-chromosomal-linked opsin genes

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Abstract

Quantitative Southern blotting and PCR/RFLP analysis were used to determine the number and ratio of long-wave-sensitive (L-) and mid-wave-sensitive (M-) opsin genes in 25 colour-normal caucasian males. The average observed ratio was 1:2.8 ± 1.2 for Southern blot analysis and 1:3.0 ± 1.7 for PCR/RFLP analysis. Thus, the two techniques yielded similar results for the ratio of L- to M-opsin genes (Wilcoxon *t*-test, $P < 0.01$). PCR/RFLP analysis of a Sma I polymorphism specific for the most proximal opsin gene suggested an average gene number of 6.0 ± 2.1, with a range from 4 to 12 in individual subjects. In contrast, Southern blot analysis suggested an average number of 3.8 ± 1.2, with a range from 2 to 7 (on the assumption that only one L-opsin gene is ever present). Differences between the L- to M-opsin gene ratio and the total gene number in some subjects may result from the presence of multiple L-opsin genes and/or hybrid opsin genes in colour-normal males. An exact determination of the total gene number will require employing other molecular techniques.

Keywords: Gene; Opsin; X-chromosome

1. Introduction

The genes encoding the human long-wave-sensitive (L-) and mid-wave-sensitive (M-) cone pigments reside in a 5′→3′ head to tail tandem array on the q-Arm (Xq28) of the X-chromosome [1]. Although it is generally accepted that, in colour-normal subjects, the most proximal position in the gene array is occupied by a L-opsin gene [1], the size and composition of the gene array is controversial.

Three molecular techniques have been used to determine the number and ratio of L- and M-opsin genes: (i) Restriction fragment length polymorphism (RFLP) quantitation after Southern-blot hybridisation [1]; (ii) RFLP quantitation of end-labelled polymerase chain reaction (PCR) products [2] and (iii) sizing of a Not I fragment that carries the entire visual pigment gene array [3]. Different conclusions about the number and ratio of L- and M-opsin genes have been drawn from these methods. Results from RFLP quantitation after Southern blot hybridisation [1,4] and Not I fragment sizing [3,5] suggest that the vast majority of colour-normal males carry a single L-opsin gene and rarely more than three M-opsin genes. For instance, Nathans et al. [1] found an average of 3.1 ± 0.6 genes in a group of 18 subjects (with no subject having more than 3 M-opsin genes); Drummond-Borg et al. [4] found an average of 3.2 ± 1.0 genes in a group of 134 subjects (with some subjects having as many as 5 M-ospin genes); and Macke and Nathans [3] found 2.9 ± 0.94 genes in a group of 67 subjects (with some subjects having as many as 4 M-opsin genes).

On the other hand, analysis of male subjects by RFLP quantitation of end-labelled PCR yields a significant higher average gene number and suggest that almost half of the subjects carry two or more L-opsin genes [2,6]. Neitz and Neitz [2] found an average of 4.3 ± 1.9 genes in a group of 27 subjects (with two observers having as many as 4 L- and 2 M-opsin genes and others having 2 L-opsin genes and as many as 7 M-opsin genes); and Neitz et al. [6] found an average of 4.48 ± 1.9 genes in a group of 26 subjects (with at least nine subjects having two or more different L-opsin genes and one subject having 9 M-opsin genes).

The significant differences between the conclusions of the three methods are troubling for our understanding

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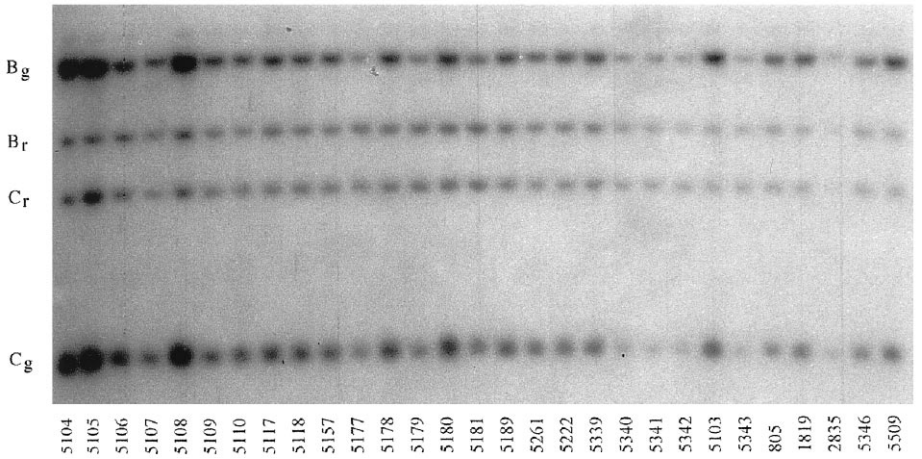

Fig. 1. Southern blot of the EcoRI/BamHI-restriction assay according to the procedure of Nathans et al. [1]. B_r and C_r are the L-opsin specific fragments; B_g and C_g, the M-opsin specific fragments. The individual samples are identified by the laboratory encoding number.

of the basic composition of the L/M pigment gene array and its variability in the human population. They also have consequences for current models of the evolution and physiology of human colour vision (see ref. [3]). To help clarify the matter, we are applying standard and newly developed molecular techniques to the same sample of 25 colour normal male subjects. A detailed report of the complete study, including all data and a comprehensive discussion of the reliability of currently applied molecular techniques, will be given elsewhere. As an interim report, here we compare the results from the two most commonly employed techniques, which are the basis for the current controversy: RFLP quantitation after Southern blotting and RFLP quantitation of end-labelled PCR products. In applying these techniques, great care was taken to reproduce faithfully the technical conditions as outlined in the previous investigations [1,2].

We find that quantitative Southern blotting and PCR/RFLP analysis yield comparable results with respect to the ratio of the L- to M-opsin genes, but differ in their estimates of the total numbers of L- and M-opsin genes.

2. Methods

2.1. Subjects and DNA extraction

Venous blood samples were obtained from 25 male subjects and total genomic DNA extracted according to the method described by Miller et al. [7]. They were all Caucasians living in southern Germany. All subjects had normal trichromatic colour vision, as verified by the Ishihara pseudo-isochromatic plates (11th Edition) and by the Rayleigh equation on the Nagel Type I anomaloscope (Schmidt and Haensch, Germany).

2.2. RFLP quantitation by Southern blot-hybridisation [1]

Total genomic DNA (10 μg) was digested with EcoRI and EcoRI/BamHI, respectively, and separated on agarose gels. DNA was transferred on positively charged nylon membranes using a pressure-blotter and fixed by uv-crosslinking. As a probe, a 350 bp EcoRI/BamHI L-opsin DNA fragment was labelled with α-^{32}P-dCTP by random priming. Hybridization was done in Hybrisol XR (Oncor) for 3–24 h at 42°. Final stringent wash was done in 0.1 X SSC, 0.1 X SDS at 65°. Hybridization signal quantitation was accomplished using a Phospho-Imager (Fuji-Bas). A blot from the EcoRI/BamHI restriction assay is shown in Fig. 1.

2.3. RFLP quantitation of end-labelled PCR fragments [2]

Two homologous segments of the L- and M-opsin genes were PCR amplified with primer pairs according to Neitz and Neitz [2]. PCR fragments were purified on agarose gels, end-labelled with T4 polynucleotide kinase and [γ-^{32}P]dATP and repurified with Qiagen spin columns and ethanol precipitation.

The end-labelled PCR fragments were digested with SmaI (183 bp promotor fragment) or Csp6I (exon 5 fragment) and separated on non-denaturating polyacrylamide gels. Quantitation of the labelled restriction fragments was done using a Phospho-Imager (Fuji-Bas).

2.4. Statistical analysis

A non-parametric test, the Wilcoxon-*t*-test [8], was used to compare statistically the mean values yielded from quantitative Southern blot analysis and quantitative RFLP analysis of the end-labelled PCR products.

3. Results

3.1. The ratio of L- to M-opsin genes

The ratio of L- to M-opsin genes was determined from quantitative Southern blot analysis of EcoRI (fragments A_r/A_g; [1]) and BamHI/EcoRI (fragments B_r/B_g and C_r/C_g) digests (for fragment nomenclature, see [1]) and compared with results from quantitative Csp6I-RFLP analysis of end-labelled PCR fragments covering exon 5 of the M- and L-opsin genes. The analyses were repeated twice in separate experiments (mean values are given in Table 1). Ratios determined from Southern blot analysis of fragment pairs, A_r/A_g, B_r/B_g and C_r/C_g, were similar for a given subject and were used to calculate an average Southern blot L- to M-opsin gene ratio. L- to M-opsin gene ratios among the 25 analysed individuals ranged between 1:1.02 and 1:5.9 with an average of $1:2.8 \pm 1.2$. A slightly higher average ratio of $1:3.0 \pm 1.7$ for all subjects (range 1:0.71–1:6.65) was found with the Csp6I-RFLP assay of end-labelled PCR products. A Wilcoxon-t-test revealed no significant difference ($P < 0.01$) between the results provided by the two methods.

However, in some samples (i.e. 5103, 5109 and 805), there were large differences in the L- to M-opsin gene ratio obtained with the two methods (see Table 1). For example, for sample 5103 the ratio was 1:4.04 for Southern blot analysis and 1:1.50 for the Csp6I-RFLP assay. Since the Southern blot analysis employed is specific for the 5′ part of the genes and the Csp6I-RFLP analysis covers only exon 5, differences found between the two assays may be explained by the presence of L-M hybrid opsin genes.

3.2. Total gene number

If one assumes the presence of a single L-opsin gene, the results of the Southern blot analysis suggests an average opsin gene number of 3.8 ± 1.2.

Direct determination of the actual opsin gene number is possible employing a SmaI polymorphism in the 5′ noncoding region, specific for the most proximal opsin gene. PCR amplification with primers specific for sequences of the most proximal opsin gene in 50 controls confirm the absence of this SmaI site (data not shown). By employing primers that enable amplification of fragments of the proximal as well as the distal pigment gene(s), quantitative comparison of SmaI restriction products should yield ratios between the most proximal gene and all other downstream pigment genes [2]. Using this assay, we found an average value of 6.0 ± 2.1 opsin genes in the gene array.

However, in some experiments, we found differences in the obtained ratios for a given sample. These differences may result from incomplete restriction, the formation of heteroduplexes or artefactual bands in unpurified PCR fragments. Moreover, in repeated dilution/re-amplification experiments, we found that there were slight differences in the amplification efficiency between the fragment derived from the most proximal opsin gene and the fragment derived from the downstream genes. PCR-amplification of exon 5 fragments of the L- and M-opsin genes in samples with one L- and one M-opsin gene revealed that the number of amplified M-opsin fragments increases 1.2–1.5 times per 25 cycles of PCR (data not shown). Nonetheless, even after taking into account a similar amplification bias for the 5′ fragments, the SmaI-RFLP assay suggests on average a higher number of opsin genes than the pure ratios of L- and M-opsin genes determined by Southern blotting or Csp6I-RFLP assays.

Nathans et al. [1] found that hybridization signal intensities of fragments A_g, B_g and C_g are either one, two or three times stronger than the signals for fragments A_r, B_r and C_r. Finding whole number ratios, they assumed that there is always a single L-opsin gene and that the total opsin gene number can be directly in-

Table 1
The relative ratio of L- to M-opsin genes and the total gene number as estimated by quantitative Southern blotting [1] and PCR/RFLP analysis [2].

Sample	Ratio of L- to M-opsin genes		Total number of genes	
	Southern blot	PCR/RFLP	Southern blot	PCR/RFLP
5103	1:4.04	1:1.50	5.04	6.30
5104	1:5.90	1:6.65	6.90	—
5105	1:3.16	1:3.12	4.16	—
5106	1:2.90	1:3.33	3.90	4.90
5107	1:2.21	1:2.71	3.21	4.26
5108	1:4.07	1:6.45	5.07	—
5109	1:2.17	1:0.74	3.17	4.50
5110	1:2.93	1:3.28	3.93	5.73
5117	1:3.13	1:0.71	4.13	8.37
5118	1:2.36	1:3.97	3.36	6.00
5157	1:2.14	1:3.09	3.14	5.97
5177	1:1.35	1:1.41	2.35	3.06
5178	1:2.82	1:2.92	3.82	5.44
5179	1:1.06	1:1.26	2.06	3.15
5180	1:2.79	1:2.96	3.79	4.02
5189	1:3.96	1:4.35	4.96	9.88
5261	1:2.99	1:4.09	4.38	4.22
5222	1:3.38	1:3.09	3.99	7.32
5339	1:1.62	1:2.02	2.62	6.27
5340	1:1.17	1:1.33	2.17	5.19
5341	1:1.02	1:1.36	2.02	4.49
5342	1:5.27	1:6.35	6.27	11.68
5343	1:2.63	1:2.86	3.63	7.01
805	1:3.08	1:1.50	4.08	7.58
1819	1:3.02	1:4.07	4.02	5.98

The Southern blotting estimates are derived from the mean values of fragment pairs A_r/A_g, B_r/B_g and C_r/C_g.

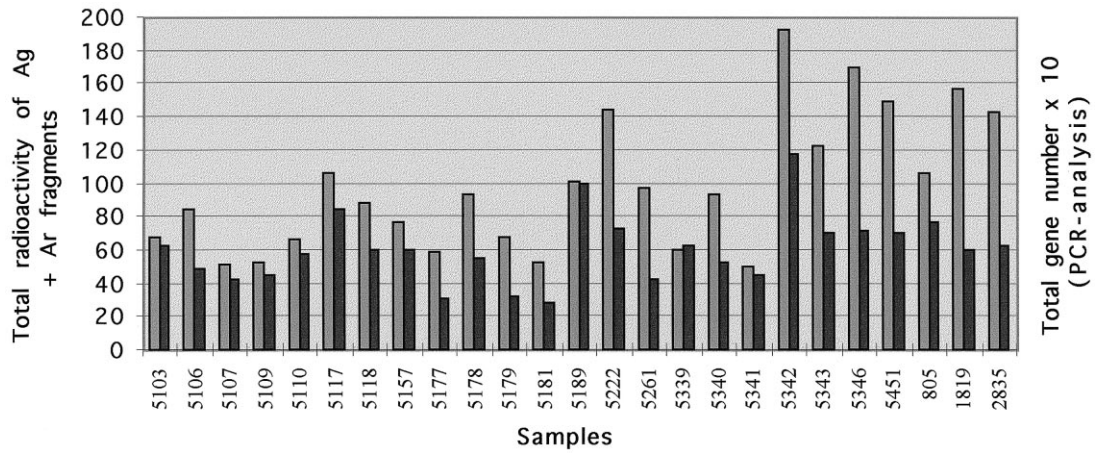

Fig. 2. Comparison of the total radioactivity of the fragment pairs $A_r + A_g$ per sample from Southern blot analysis (left hand bars) and the total number of the X-linked pigment genes from the Sma I-RFLP-analysis (right hand bars, multiplied by 10 for clarity).

ferred from the L- and M-opsin gene ratio. Using sensitive and linear responsive phospho-imaging analysis of Southern blots, however, we could not confirm only whole number ratios. This suggests that at least in some subjects there may be more than one L-opsin gene and/or L-/M-hybrid opsin genes present in the gene array of colour-normal males [2,6]. This assumption is supported by the observed tendency of higher combined signal intensities of L- and M-opsin specific fragments in the Southern blot analysis-independent of the actual L-/M-opsin gene ratio in samples that also yield higher ratios in the SmaI-RFLP assay (Fig. 2).

4. Discussion

The discrepant results obtained from different molecular methods used to determine the number and ratio of X-linked pigment genes has raised the question about the reliability of the employed methods. Macke and Nathans [3] recently reported that results from Southern blotting and pulsed field gel electrophoretic sizing of Not I fragments yield a comparable spectrum of opsin gene numbers, but these values differ significantly from the opsin gene numbers determined by the RFLP-analysis of end-labelled PCR products [2]. Here we report that Southern blot hybridization and RFLP analysis of end-labelled PCR product-analysis, done on the same set of samples-yield similar ratios of L- to M-opsin genes, but different values of the total opsin gene number in the array. Thus using the same assays as employed in previous studies, we were able to reproduce the discrepancies found between those studies [2], in the same group of observers. To resolve the matter further, we are currently extending our study, to a larger group of colour normal male subjects, employing direct pulsed-field gel electrophoresis as well as other molecular techniques.

Here we will only briefly consider some sources of variability that might account for the discrepancies between the Southern blotting method and the PCR-analysis. First, we find that the PCR based RFLP system may over-represent the number of M-opsin genes by a factor of 1.2–1.5 because of unequal amplification of the corresponding opsin gene sequences. This was revealed by a repeated amplification/dilution of probes that showed an initial one to one ratio by the two methods. This fact may explain the slightly increased values for the number of the M-opsin genes (as well as the higher number of genes in the array) derived from the Csp6I-RFLP analysis as compared with Southern blot hybridization (Table 1). Additionally, we were unable to resolve with sufficient reproducibility heteroduplexes in the PCR/RFLP assay. This may be a source of incorrect quantification as well as incomplete restriction enzyme digestion.

Second, as described above, Southern blot hybridization does not directly determine the total number of the X-linked pigment genes. Nathans et al. [1] found increased hybridization signals for the M-opsin-specific fragments compared with the L-opsin-specific fragment signals in unit steps. Based upon the assumption that there is only one L-opsin gene per X-chromosome, the hybridisation signals of the fragment pairs (A_r/A_g, B_r/B_g and C_r/C_g) should increase linearly with an increase in the number of M-opsin genes per sample, whereas the signal strength of the L-specific fragments should always be equal. However, if increased hybridization signals for the fragment pairs as well as increased signals of the L-cone specific fragments are observed in a sample and if PCR-analysis indicates a high number of opsin genes, as found in this study, the existence of more than one L-opsin gene in the sample may be implied.

How can the presence of multiple L-opsin genes in the array be explained? Multiplicity of the M-opsin genes has been explained as resulting from unequal

recombination between genes located at different positions within the array [1]. However, the L-opsin gene is located at the proximal end of the gene array. Thus a transfer of a complete L-opsin gene as the result of an unequal recombination event is unlikely. There is, however, the possibility of intragenic exchange resulting in the formation of a M1L2 (containing exon 1 of the M-cone pigment gene and exons 2–6 of the L-cone pigment gene) or M2L3 hybrid-gene in the second or third position of the array. A M1L2 hybrid gene, for instance, would be a *de facto* L-opsin gene (i.e. indistinguishable from it) because of the complete sequence homology of exon 1 in the M- and L-opsin gene types.

In conclusion, we show that previously reported discrepancies in the determination of the number and ratios of L- and M-opsin genes can be reproduced when the two most common technical approaches are applied to the same sample of observers. Further investigations and the implementation of new, more direct techniques will be necessary to resolve this problem.

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